Latex and Leaf Venation Networks: Coevolution of Laticiferous Plants with Simulated Insect Herbivory

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ABSTRACT

Plants and insects have coevolved and developed intricate relationships for millions of years. Latex exudation and insect herbivory is a prime example of one such relationship. Latex plays a defensive role in plants, as it is exuded from the leaf due to damage, and coagulates upon exposure to air. This characteristic makes it difficult for insects to feed on plant leaves, which are a primary site for insect herbivory. Latex producing plants are found in more than 40 families, and are thought to have been independently evolved multiple times. The laticifers, cells which contain and move latex throughout a plant, can elucidate the inner workings and function of latex producing plant defense systems. Laticifer occurrence at different scales within the leaf and its impact on plant growth and development also remain to be investigated. In this study, latex exudation was measured (in mm^3) for different latex producing plants across three vein hierarchies (midrib, secondary, tertiary/lamina). Different treatments were applied: single site damage, proximal midrib damage, and proximal whole trench. The response to these treatments was measured for the three different hierarchies. The results indicate that at every midrib and secondary vein, there is a laticifer network present, and that latex amounts decrease from midrib, secondary, and tertiary/lamina veins respectively, for most species. Additionally, latex amounts remaining after proximal midrib and whole trench damage are lower than those without any proximal damage. Together, these results indicate that venation network hierarchy plays both a functional and defensive role in plants.

KEYWORDS

Latex exudation, laticifers, plant defense, trenching behaviors, vein hierarchies

INTRODUCTION

Plants have evolved many different defense systems to promote leaf survival, because leaves are one of the main focus of herbivory and are a primary site of infection by microorganisms. One form of plant defense against herbivory is the release of exudates, of which latex is the most common type. Latex is a glue-like substance and usually has a milky appearance, but can be colorless and difficult to visualize (Agrawal and Fishbein 2008). Latex is highly variable in its chemical composition, and is known to have toxic properties against insect herbivory. The vast number of cysteine proteases, along with the chitinases, lectins, lipases, oxidative and other hydrolytic enzymes within latex are used for plant defense (Ramos et al. 2010). Latex is stored in specialized plant cells called laticifers, which play a major role in the production and transport of latex (Pickard 2008).

Counteradaptation of insects to latex defense systems have evolved in intricate and dynamic ways. Plants and insects have had over 350 million years of shared history and coevolution (War et al. 2012). Insects have evolved ways to counteract latex via trenching and, the oldest known fossil of latex-sabotaging insect behavior is seen in the Eocene, about 55.8 to 33.9 million years ago, from brown coals in Geiseltal, Germany (McCoy et al. 2022). The vein cutting behavior of certain insects is a strategy that is used to sever veins to block the flow of latex to feeding sites (Clarke and Zalucki 2000, Dussourd and Denno 1991, Dussourd and Eisner 1987, Helmus and Dussourd 2005). This counteradaptation to avoid latex is partly due to the sticky nature of latex; when the tissues of latex producing plants are damaged the latex oozes out, becomes sticky when exposed to air, and quickly coagulates (Agrawal and Konno 2009). Visualizing latex within plant leaves has not been widely studied, though it is a key step to comprehending the relationship between latex producing plants and herbivorous insects.

Latex is found in plants of more than 40 families, and over 20,000 species are thought to bear laticiferous structures (Lewinsohn 1991). This diversity of latex producing species suggests that latex production is a highly convergent trait, and has been independently evolved multiple times (Agrawal and Konno 2009). Additionally, latex composition can be diverse between closely related species in the same family or genera; yet latex composition of plant species that are distant in phylogeny are often similar (Konno 2011). This further indicates latex is a

convergent trait. This repeated evolution suggests that the innovation and adoption of latex by plants confers a defense benefit (Farrel et al. 1991).

Similar to latex, the vein-cutting and trenching behaviors of herbivorous insects have evolved independently multiple times in at least three orders of insects (Agrawal and Konno 2009). Trenching is a strategy used by insects to reduce latex at feeding sites by cutting all vein hierarchies.

A latex producing plant has one of two distinct types of laticifers: articulated or non-articulated laticifers. Non-articulated laticifers originate from single cells with reticulate branching. Conversely, articulating laticifers are compound in origin and have rows of cells that are derived from the surrounding phloem tissues (Castelblanque et al. 2017). Laticifers tend to closely follow the pattern of veins within a leaf (Blaser 1945). *However, it is unknown how laticifer spread and extent vary from venation network depending on species or vein architecture.*

Variation between leaves of different species is common. For example, leaf shape is known to depend on at least two homeobox genes, and shape varies tremendously among species (Kierzkowski et al. 2019); leaf sizes can vary from less than 1 mm^3 to greater than 1 m^2 in area (Wright et al. 2017). Vein traits across species vary; larger leaves have major veins with larger diameters, but lower major vein density. Total leaf vein density and minor vein traits are typically independent of leaf size among both palmate and pinnate species (Sack et al. 2012). Leaf venation networks also show variation in their geometry, with some that form extensive branching and reconnecting networks (Roth-Nebelsick 2001) and others do not. Leaf vein networks have evolved defensively to counter attacks from predation and herbivory. For example, developing high vein density and reticulation (Blonder et al. 2018, Read and Stokes 2006) may cause insects to expend more energy cutting through clusters of veins, thereby providing an advantage against herbivory.

Understanding the spatial distribution of latex within a leaf and how it is impacted by herbivory means that visualization methods must be applicable to the wide variety of latex producing plants, and their many differing traits. Not many methods exist to visualize the laticifers within plant leaves and observe their relationship with venation network pathways. As a result few plant species have been studied in this area. This lack of knowledge for latex producing plants is the motivation for this study.

Here, I ask how the distribution of latex within a leaf is impacted by simulated insect herbivory, and how latex production is related to the location of laticifers within the leaf venation system. To understand the impact of latex production on herbivory, I must first assess the distribution and flow of latex within a plant leaf. Therefore I ask these three questions:

1. Does every location in the leaf that has a vein also have a laticifer, or is the laticifer network a subset of the whole venation network?
    - H1A: Within the leaf venation network any location in a leaf that has a vein will also have a laticifer.
        - Rationale: Insects cut the veins that flow to the site of intended herbivory (Dussourd and Eisner 1987), suggesting that veins are always latex-containing.
2. How does venation network architecture influence the response of latex production in different sites when injured, across four major latex producing families (Moraceae, Apocynaceae, Asclepiadaceae, and Euphorbiaceae)?
    - H2: Across hierarchies, larger veins are expected to lose higher latex amounts than minor laticifers as latex flow is expected to be higher in larger laticifers at proximal damages sites that are close to the leaf petiole.
        - Rationale: It can be assumed that larger veins are capable of holding more latex than smaller veins due to size. This trend would likely be true for damage to leaf veins in response to processes such as trenching. To satisfy the conditions of this hypothesis, latex exudation amounts must decrease from midrib to secondary to tertiary.
3. How does proximal vein cutting/damage affect latex amounts released at distal end, across the different vein hierarchies?
    - H3A: Proximal vein damage will decrease the amount of latex exuded at the distal vein hierarchies while still maintaining midrib > secondary > tertiary/lamina exudation scales
        - Rationale: It is known that insects perform trenching behaviors at the proximal end of a leaf to decrease the amount of latex exuded at the distal end (Dussourd and Eisner 1987). To satisfy the conditions of this

hypothesis latex exudation at the distal hierarchies must be less than latex exudation at the proximal end and without prior damage.

METHODS

Study site and sampling methods

I used 29 plants from known latex producing species located in the University of California Berkeley Botanical Garden (UCBG), located on the Berkeley campus in Strawberry Canyon (37.8751° N, 122.2387° W) (Table 1). The 34-acre UCBG Garden contains a variety of latex producing plants from many global origins. After acquiring a master list of all the species in the botanical garden I searched for families that were known to contain latex and tested individual species from those families (Moraceae, Apocynaceae, Asclepiadaceae, and Euphorbiaceae) to verify if they had latex. The various latex producing plants require different growth conditions within the garden itself. For this study I tagged plants from the arid house, the garden beds, and the nursery. However, I was only able to sample the arid house plants due to methodological and logistical constraints. Each plant was required to have at least nine leaves for one full experiment. However, plants with at least three leaves were also considered for measurement of a few traits. When leaves were in excess, the additional leaves could be used for replications and as a buffer for potential errors.

Table 1. List of all plant species tested in this study and their abbreviations and family.

Species name	Abbreviation	Family
Pachypodium baronii	Pac_bar	APOCYNACEAE
Fockea edulis	Foc_edu	APOCYNACEAE
Pachypodium lealii	Pac_lea	APOCYNACEAE
Pachypodium rosulatum	Pac_ros	APOCYNACEAE
Pachypodium saundersii	Pac_sau	APOCYNACEAE
Fockea sp.	Foc_spp	ASCLEPIADACEAE
Fockea gracilis	Foc_gra	ASCLEPIADACEAE

Gonolobus gonocarpos	Gon_gon	ASCLEPIADACEAE
Euphorbia hofstaetter	Eup_hof	EUPHORBIACEAE
Synadenium molle	Syn_mol	EUPHORBIACEAE
Euphorbia monteiroi	Eup_mon	EUPHORBIACEAE
Euphorbia neohumbertii	Eup_neo	EUPHORBIACEAE
Euphorbia vigueri	Eup_vig	EUPHORBIACEAE
Euphorbia perrieri var. elongata	Eup_per	EUPHORBIACEAE
Euphorbia capuronii	Eup_cap	EUPHORBIACEAE
Euphorbia neriifolia cv. cristata	Eup_ner	EUPHORBIACEAE
Euphorbia milii var. bosseri	Eup_mil	EUPHORBIACEAE
Euphorbia bupleurifolia	Eup_bur	EUPHORBIACEAE
Euphorbia invenusta	Eup_inv	EUPHORBIACEAE
Euphorbia duranii var. ankaratrae	Eup_dur	EUPHORBIACEAE
Euphorbia croizatii	Eup_cro	EUPHORBIACEAE
Euphorbia milii var. spendens	Eup_mil_vs	EUPHORBIACEAE
Euphorbia decaryi var. cap-saintemariensis	Eup_dec	EUPHORBIACEAE
Monadenium rhizophorum var. stoloniferum	Mon_rhi	EUPHORBIACEAE
Euphorbia socotrana	Eup_soc	EUPHORBIACEAE
Ficus brandegeei	Fic_bra	MORACEAE
Ficus palmeri	Fic_pal	MORACEAE
Dorstenia gigas	Dor_gig	MORACEAE
Dorsentia foetida	Dor_foe	MORACEAE

Latex visualization methods tested

Laticifer pathways visualization in leaves is a difficult process. There are few published reports of methods for latex visualization, e.g. by Oppel et al. (2009) and Castelbanque et al.

(2016). My initial research endeavors involved testing these two visualization techniques across a diverse range of latex-producing plants. Unfortunately, these methods were not generalizable to the species studied here.

Microwave-assisted extraction technique (Oppel et al. 2009)

The Oppel et al. (2009) method employs microwave-assisted extraction to visualize the alkaloids found in latex. This method is meant to visualize the alkaloids in a mature leaf, visualizing the latex and therefore laticifer pathways. To perform this method I cut and cauterized the leaf with a heated blade to minimize latex loss. I partially dried the leaf in an oven set to 50 °C and sandwiched it between polyester backed silica gel Thin Layer Chromatography (TLC) plates treated with toluene. I placed this assembly in a microwave oven under a weighted dish (4.4 kg) and microwaved it at 30% power for 11 minutes. After microwaving, the assembly was left to cool down for about 10 minutes before being taken out and the TLC plates sprayed with freshly prepared Dragendorff's reagent (bismuth(III) nitrate ($Bi(NO_3)_3$), Hydrochloric acid 30-40%, and potassium iodide 99.8% (KI)). Finally, the plates were left out to develop for up to an hour. Over the span of 29 trials the microwave assisted extraction technique was unsuccessfully applied to 14 new species (Figure 1). The microwave-assisted extraction technique had only previously been used on mature *Lobelia cardinalis* leaves, not on any other species. I determined that the microwave-assisted extraction technique may be incapable of visualizing the wide variety of latex producing plants being studied.

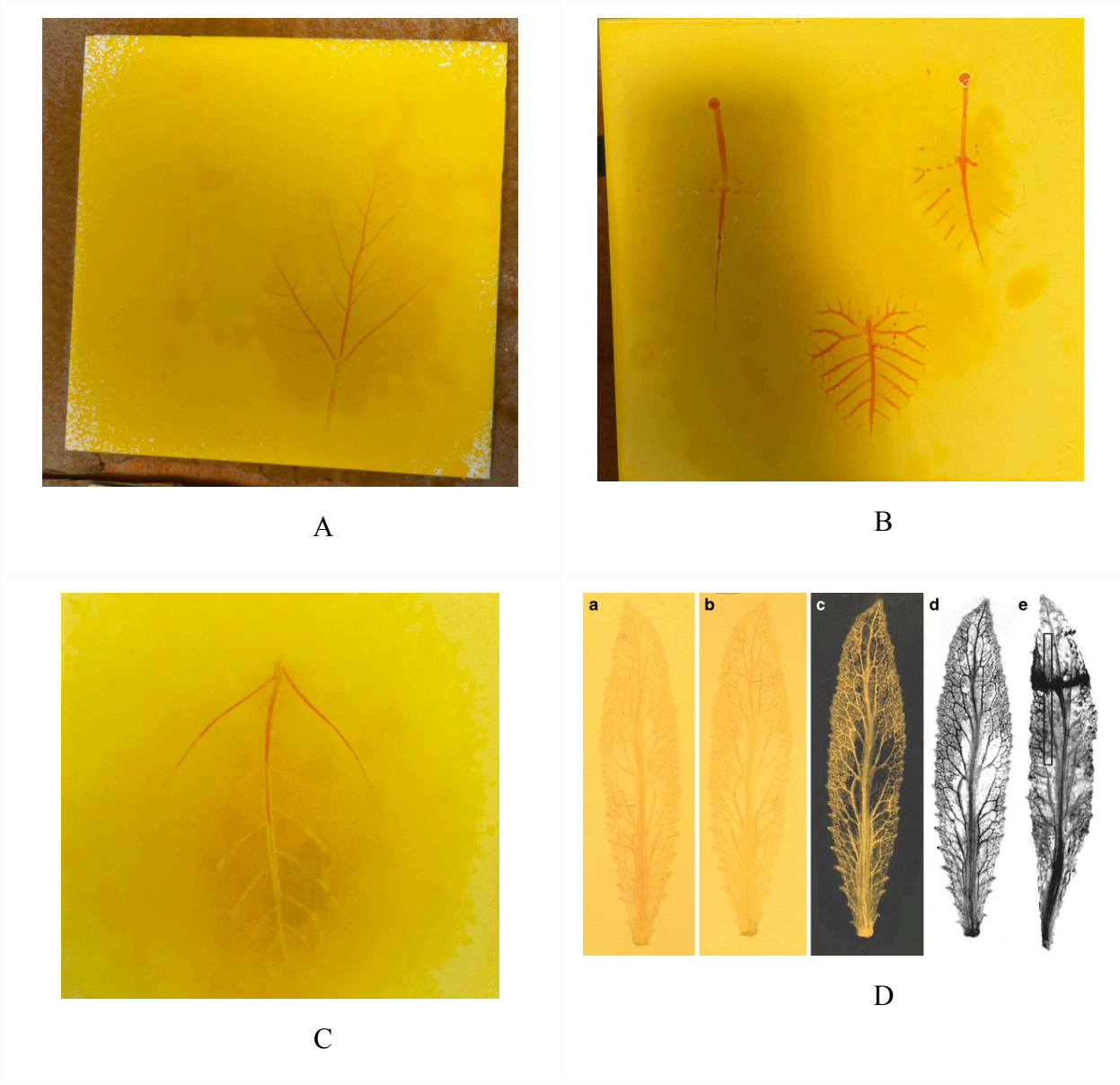


Figure 1. Microwave-assisted extraction trials. Results of the Oppel et al microwave-assisted extraction technique tested on three Moraceae species alongside the original Oppel image tested on *Lobelia cardinalis*. (A) *Ficus carica* (fig tree) (Moraceae). (B) *Ficus palmeri* (Moraceae). (C) *Ficus carica* (fig tree) (Moraceae). (D) Figure 2 from Oppel et al., 2009; Alkaloid distribution in *Lobelia cardinalis* leaves as visualized by the microwave extraction procedure.

Sudan Black B staining (Castelblanque et al. 2016)

Castelblanque et al. (2016) aimed to stain alkaloids within plant leaves using Sudan Black B dye, followed by a clearing process that leaves only the laticifer pathways. The Sudan Black B dye was a filtered solution of 0.7g of Sudan Black B powder dye + 100 mL Propylene

glycol. My attempts to replicate the results of the Castelblanque et al. paper closely adhered to their methods. I collected plant leaves and cauterized them with a heated blade to reduce latex loss. Then I washed the leaf with 70% ethanol to remove any surface impurities, and stained the leaf for up to 3 hours with Sudan Black B dye. I then washed the leaf with 70% ethanol again. Finally, the leaf was washed with water for 3-5 minutes and placed in a 2.5M NaOH solution for hours to multiple days to be cleared. Leaf veins could not be adequately stained by the Sudan Black B, therefore clearing the leaf did not leave visual traces on laticifers. Unfortunately, repeated difficulties with the leaf staining and clearing led me to abandon this visualization method. Castleblanque et al. used the Sudan Black B dye on the leaves of seedlings. As seedlings have soft tissue they can easily absorb the Sudan Black B dye into their veins. However, when attempting to dye mature leaves with already lignified veins, as used in this study, the Sudan Black B absorption seems to become difficult to near impossible. The Sudan Black B staining technique was unsuccessfully applied to mature leaves of 2 new species over the span of 11 trials (Figure 2).

A 

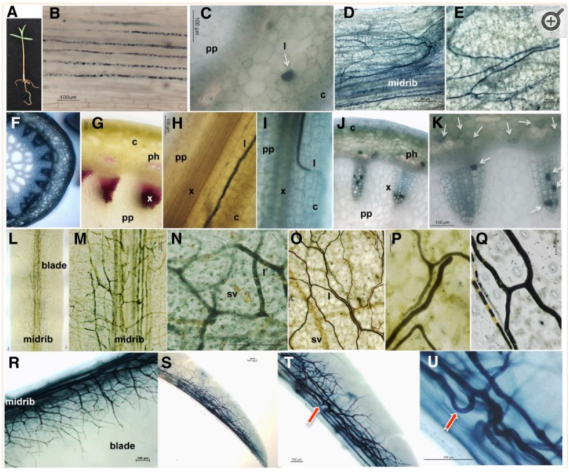
B 

Figure 2. Sudan Black B staining technique. (A) *Ficus carica* leaf set in Sudan dye as a branch for 25 minutes, extra 10 minutes fully submerged. (B) Figure 1 from Castelblanque et al., 2016. Distribution pattern of laticifer cells in *E. lathyris* intact plant structures as revealed by whole-mount staining with Sudan Black B.

These two methods did not provide sufficient data to visualize laticifer pathways in the wide variety of latex producing plants. This lack of generalizability ultimately led to the

development and implementation of the razor blade method to measure latex exudation in relation to laticifer pathways.

Study method

Instead of visualizing the complete laticifer network, I verified its presence/absence in specific locations by making small cuts into the leaf. This experiment is not a direct herbivory behavior, but mimics a way in which insects could damage a leaf. Although this method allows for the direct assessment of latex exudation volume with the benefit of having an intact connection with the rest of the plant, it does not allow for a full visualization of the laticifer network. Additionally, this method is highly destructive due to the cutting method that physically damages the leaf.

I used this method for three tests; each run on separate leaves of the same plant. There are three leaves required per test, so a total of 9 leaves are required to collect a complete dataset for a given species. Each leaf that is tested is first measured with a ruler (Figure 3A) and divided into 4 sections, the black dots represent the 25%, 50% and 75% divisions of the leaf when going from petiole to tip (Figure 3B). The midrib is the thickest vein along the midline of a leaf. The secondary vein is the second thickest vein and comes directly off the midrib. The tertiary vein is the third thickest vein that comes off of the secondary vein and the lamina is the flat part of the leaf with chloroplasts. Tertiary/lamina are grouped together because I was unable to be precise enough with my cuts to measure the very small tertiary vein alone so it almost always includes the lamina. The proximal end is nearer to the stem and closer to the petiole of the leaf. The distal end is further from the stem and closer to the tip of the leaf.

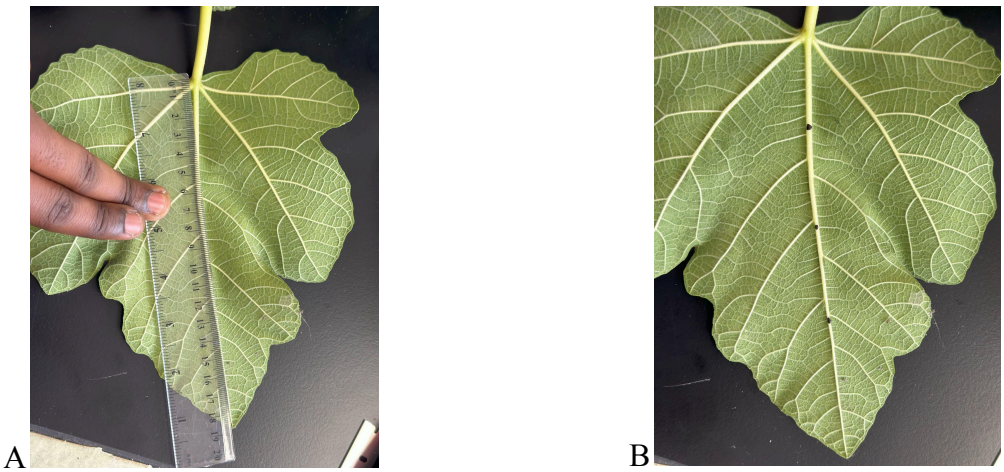


Figure 3. Leaf measurements. (A) Demonstration of how measurements for leaf length were made from petiole to tip with a ruler. This leaf is to show how measurements were made and is not attached to the plant like the study organisms were. (B) The 25%, 50%, and 75% locations of a leaf marked with sharpie along the midrib, indicating where cut sites would be located.

Individual hierarchy measurements (midrib, secondary, tertiary)

To determine if every location in a leaf which has a vein also has a laticifer, and to collect the latex exuded from each vein hierarchy, I used a razor blade to damage each vein hierarchy and visually assess if latex was exuded. Latex exudation was collected using a microcapillary tube (Capillary Lab Tube, StonyLab, length 100mm, O.D 0.5, I.D 0.3. NB Quantity is 1000). The razor blade protocol ensures that each vein hierarchy was tested on a different leaf, so no prior intended damage influences the results. The cut was at the 50% mark of the leaf in regards longitudinally between the petiole and the tip. For example on one leaf, only the midrib was cut at the 50% mark, and the presence of latex was visually assessed before being collected via a microcapillary tube. The volume of latex was recorded by measuring in mm how far the latex was taken up by the microcapillary tube and multiplying by $\pi * r^2$ (where r is the radius of the microcapillary tube, here 0.15 mm). This data collection method was replicated for each vein hierarchy (midrib, secondary, and tertiary/lamina), and each species tested.

To determine if every location in a leaf that has a vein also has a laticifer and to understand if the laticifer network is a subset of the leaf venation network, I visually assessed the presence of latex at cut sites for each hierarchy. The presence of latex exudation is assessed to provide insights regarding laticifer presence/absence, as a laticifer must be present for latex to be

exuded. Observations were recorded as either “presence” or “absence” of latex exudation. The visual assessment and recording of latex presence was straightforward and allowed for comparison across species. The visual assessment allowed for the association between vein hierarchy and latex presence to be observed. Where the exudation was scored as 1 (yes, latex observed) and 0 (no, no latex observed).

To determine if the amount of latex exudation scales with the size of the vein, I cut leaves across three vein hierarchies and measured the latex exudation volume. This data provided valuable information regarding exudation amounts across different vein hierarchies. Leaf measurements were collected for length (petiole to tip), width (from one edge of the leaf to another at the 50% mark), and height of a leaf (using a digital thickness gauge); to provide approximations for leaf area (length * thickness).

Damage response

To determine the distal latex exudation at different hierarchies in response to proximal vein cutting/damage, I cut veins with a razor blade. This entailed cutting the proximal midrib at the 25% mark of the leaf (closest to the petiole) and measuring exudation volume with a microcapillary tube. Twenty seconds after the proximal cut, another cut was made at the distal end at the 75% mark of the leaf (closest to the tip) and the exudation volume was measured with a microcapillary tube. The distal cuts were for the midrib, secondary, and tertiary/lamina. Each different hierarchy was tested on a separate leaf, all with proximal midrib damage.

To measure the response of latex exudation at different hierarchies to trenching, I used a razor blade and mimicked trenching by cutting all proximal vein hierarchies. I used a razor blade to cut all vein hierarchies at the proximal 25% mark on the leaf. I cut from one side of the leaf to the other, trying not to sever the midrib. I waited 20 seconds after the trench to make a cut at the distal 75% mark on the leaf after trenching. Latex volume was only recorded for the cuts at the distal end after trenching. The distal cuts were the midrib, secondary, and tertiary/lamina locations on different leaves that each had proximal trenching. The different cut sites were abbreviated for ease of reference (Table 2). Six leaves were required to perform these two damage tests, and determine the influence of proximal vein cutting and whole trench damage on distal latex production/exudation.

Table 2. List of all cut site references in this study and their abbreviations.

Cut site	Abbreviation
midrib	m
secondary	s
tertiary/lamina	t
midrib proximal	m_p
midrib distal	m_d
secondary distal	s_d
tertiary distal	t_d
whole trench midrib distal	wt_md
whole trench secondary distal	wt_sd
whole trench tertiary distal	wt_td

Statistical analysis

Research Questions

Research question 1, latex presence/absence. I counted the fraction of species that had latex at each vein size to summarize the results. There were 29/29 latex presence scores for midrib and secondary tested species, and there were 27/28 scores for tertiary/lamina tested species.

Research question 2, vein hierarchy exudation. I calculated the average exudations for the midrib, secondary, and tertiary/lamina to compare exudation amounts across hierarchies. This was done by adding up the total exudation from all species and dividing by the total number of species tested for that hierarchy. Additionally, figures were made in R to display exudation results across hierarchies.

Research question 3, damage response. I analyzed damage responses to proximal midrib damage and whole trench damage. To study the response a couple of calculations were necessary, examples of these equations are in parenthesis. Calculations were done in R.

- The difference between the undamaged hierarchy and the distal hierarchy after proximal midrib cut (m - m_d).
- The difference between proximal midrib and distal hierarchy (m_p - m_d).
- The difference between the undamaged hierarchy and the distal hierarchy after trench (m - wt_md).

ANOVA and Tukey test

To compare the differences between damage treatments an analysis of variance (ANOVA) was done for the midrib, secondary, and tertiary vein hierarchies, and was followed up with a Tukey test. All analysis and figures were generated in R version 4.3.2 (R Core Team 2023) using packages ggplot2 (Wickham 2009), lme4 (Bates et al. 2015), tidyverse (Wickham et al. 2019), and ggpubr (Kassambara 2009). The R code is available in the Appendix.

RESULTS

Research question 1: Laticifer presence by vein hierarchy

Latex exudation was visually assessed by individual hierarchy in various latex producing species. Out of the 29 species tested, I found that latex was present in the midrib and secondary veins of 100% of the species. I also found that 1 species of the 28 tested for tertiary/lamina veins did not exude latex when damaged, 96% of the species had latex presence (Figure 4).

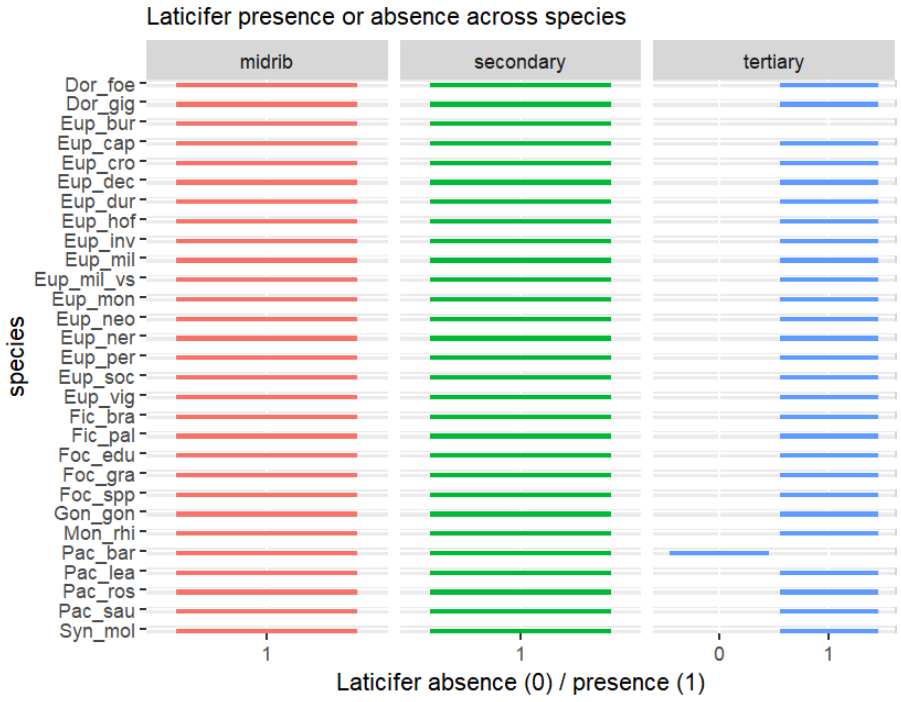


Figure 4. Presence or absence. Illustration of the visual assessment of latex exudation at the midrib, secondary, and tertiary/lamina on a basis of presence or absence. Each row shows a different species, each species is abbreviated (code in Table 1). Bars are colored if latex is present and are gray if latex is absent. Midrib is red, secondary is green, and tertiary/lamina is blue.

Research question 2: Impact of hierarchy on exudation

Latex volume varied with hierarchy. I found that the most latex was exuded from a cut to the midrib followed by the secondary vein and tertiary vein respectively. Latex exudation decreased by hierarchy; this trend was observed in multiple species of latex producing plants (Figure 5). Additionally, trends can be seen between families, which are color coded in figure C. However, some of this data may be skewed as some families were tested far more than others (Figure 5B & C)

Average exudations

On average 4.591 mm^3 latex is exuded from the midrib, 1.084 mm^3 of latex is exuded from the secondary vein hierarchy, and 0.294 mm^3 of latex is exuded from the tertiary/lamina grouping.

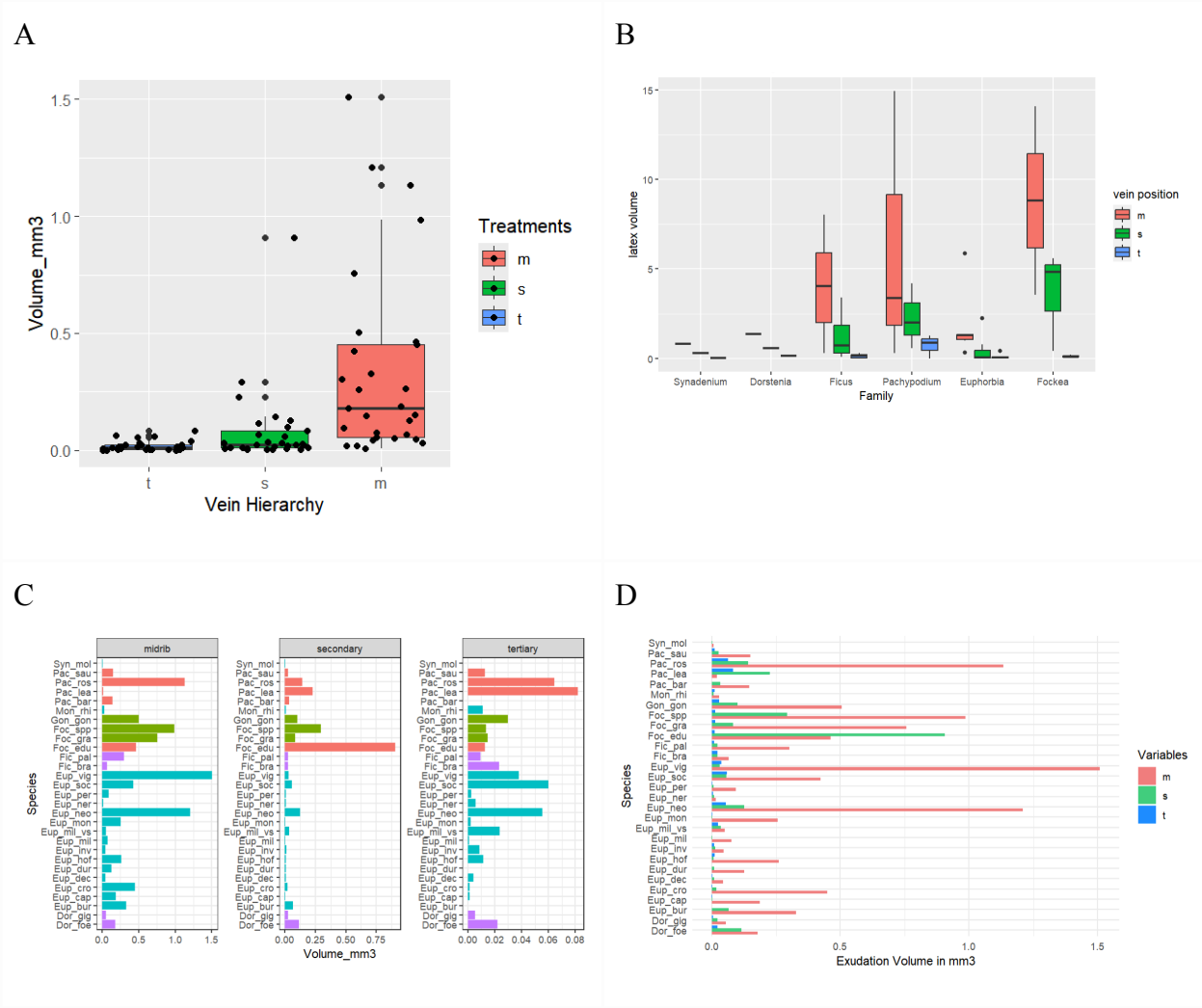


Figure 5. Latex exudation measurements. (A) Barplot showing mean latex exudation by venation hierarchy across species. (B) Variation across family for latex exudation. For figures A & B the hierarchies are color coded;l midrib in red, secondary in green, and tertiary/lamina in blue. (C & D) Latex exudation levels in midrib, secondary, and tertiary/lamina veins (bottom). In figure C the families are color coded (Euphorbiaceae in blue, Asclepiadaceae in green, Apocynaceae in red, Moraceae in purple). In figure D the colors represent hierarchies

Research question 3: Impact of damage on exudation

Proximal midrib damage and whole trench damage, when followed by measurement of latex volume at the distal hierarchies, resulted in decreased latex exudation at the distal end of the leaf. Latex exudation is lower at the distal end of a leaf, regardless of hierarchy, when compared to the proximal midrib damage exudation amount from the same leaf. This trend of

latex exudation was observed across the various species tested. The exact differences between the amount of latex exuded at the proximal and distal ends varies across species and hierarchies.

These values and calculations can be compared across hierarchies and species by calculating it into percentages and visualizing it graphically. To study damage response, I calculated the difference between the undamaged hierarchy and the distal hierarchy after proximal midrib cut, for example m - m_d. Hierarchy relationships can also be studied by calculating the difference between the undamaged hierarchy and the distal hierarchy after trench, for example m - wt_md. The difference between the damage site and damage response can be found by calculating the difference between proximal midrib and distal hierarchy, for example m_p - m_d.

Average Exudations

On average latex volume decreased by 89.51%, 6.249 mm^3 (6.981 - 0.732), for the distal midrib when compared to the proximal midrib latex exudation (m_p - m_d). On average latex volume decreased by 98.71%, 6.891 mm^3 (6.981 - 0.090) for the distal secondary vein hierarchy when compared to the proximal midrib latex exudation (m_p - s_d). On average latex volume decreased by 99.42%, 6.941 mm^3 (6.981 - 0.040) for the distal tertiary/lamina hierarchy when compared to the proximal midrib latex exudation (m_p - t_d).

On average latex exudation in the distal midrib was measured to be less than the undamaged midrib by 84.05%, 3.859 mm^3 (4.591 - 0.732) (m - m_d). On average latex exudation in the distal secondary vein hierarchy was less than the undamaged secondary vein hierarchy by 91.69%, 0.994 mm^3 (1.084 - 0.090) (s - s_d). On average latex exudation in the disital tertiary/lamina was less than the undamaged tertiary/lamina by 86.39%, 0.254 mm^3 (0.294 - 0.040) (t - t_d) .

On average whole trench midrib latex exudation is less than undamaged midrib latex exudation by 94.09%, 4.32 mm^3 (4.591 - 0.271) (m - wt_md). On average, whole trench secondary vein hierarchy latex exudation was less than undamaged secondary vein latex exudation by 88.83%, 0.963 mm^3 (1.084 - 0.121) (s - wt_sd). On average whole trench tertiary/lamina latex exudation was less than undamaged tertiary/lamina latex exudation by 96.93%, 0.285 mm^3 (0.294 - 0.009) (t - wt_td).

Overall proximal damage decreases latex capacity at the distal end of a leaf. Whole trench damage depletes more distal latex at the midrib and tertiary/lamina than proximal midrib damage. Proximal midrib damage depletes more distal latex at the secondary vein than whole trench damage. Each hierarchy and its damage response latex exudation values when viewed across species provide insights for these trends (Figure 6). The decreased distal latex exudation volume following proximal damage indicates that there is an evolutionary advantage for insects that have vein cutting and trenching behaviors (Figure 6).

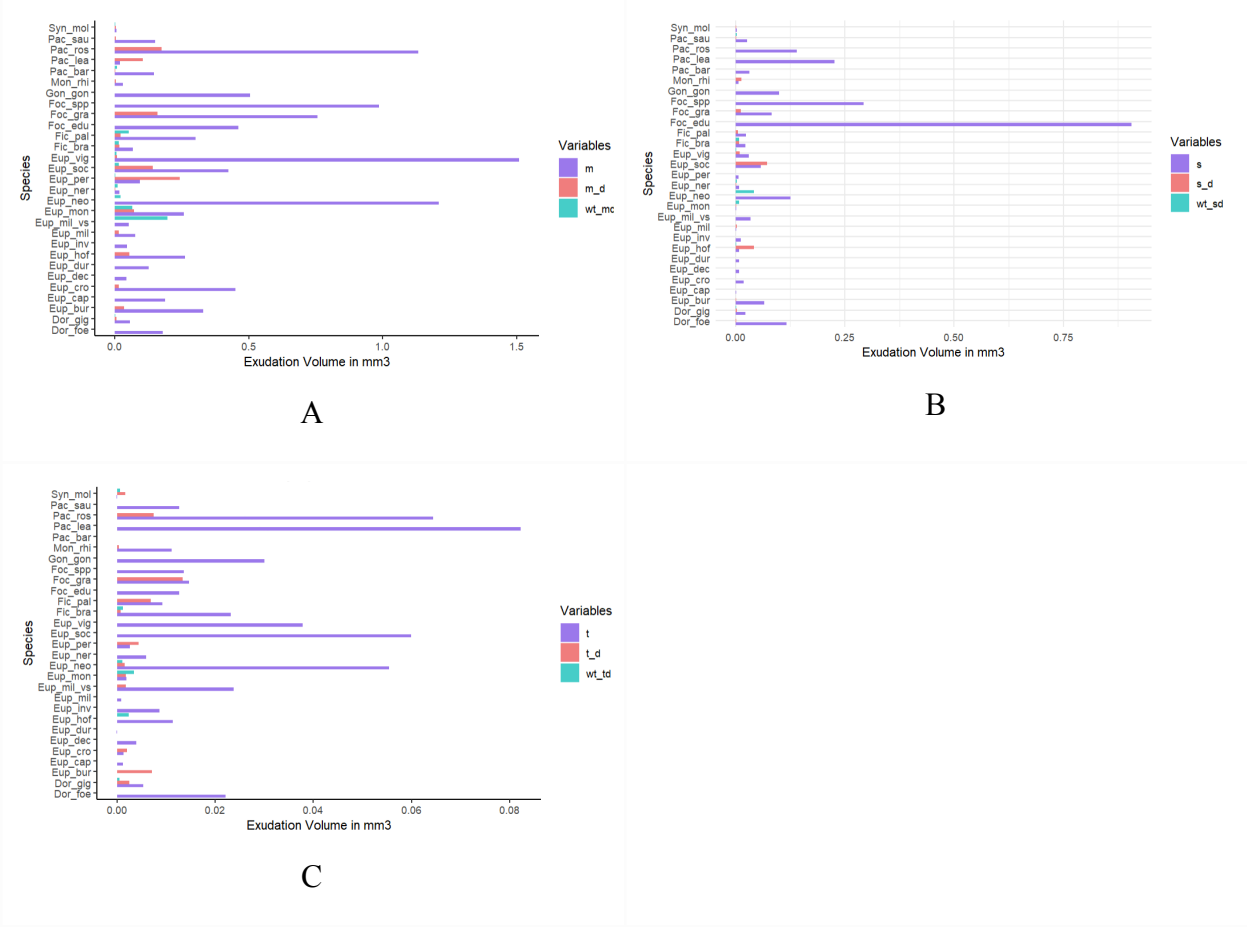


Figure 6. Damage responses. Damage to just a single hierarchy is in blue, the response to proximal midrib damage is in red, and the response to a whole trench at the proximal end of a leaf is in green. All the data used in these figures is raw data (not normalized). (A) Comparison between exudations of midrib, distal midrib to proximal midrib cut, and distal midrib to whole trench. (B) Comparison between exudations of secondary, distal secondary to proximal midrib cut, and distal secondary to whole trench. (C) Comparison between exudations of tertiary/lamina, distal tertiary/lamina to proximal midrib cut, and distal tertiary/lamina to whole trench

ANOVA and Tukey test

The results of this ANOVA means that the probability that the means are the same is false, so I reject the null hypothesis that they are the same (Table 3). The results demonstrated by the P value of $1.39 * 10^{-5}$ being considered statistically significant. The P value means that latex response to herbivory attack differs at different vein hierarchies. The results of the ANOVA led to a tukey test to determine if there is a difference between the mean of all the possible pairs (Figure 8). There is a significant difference between the secondary and midrib, and the tertiary and midrib. There is no significant difference between the tertiary and secondary.

Table 3. ANOVA. Performed in R to analyze the statistical significance of the data.

	Degrees of Freedom	Sum of Squares	Mean of Squares	F value	P value
Treatments	2	1.67	0.8348	12.84	1.38e-05
Residuals	83	5.397	0.0650		

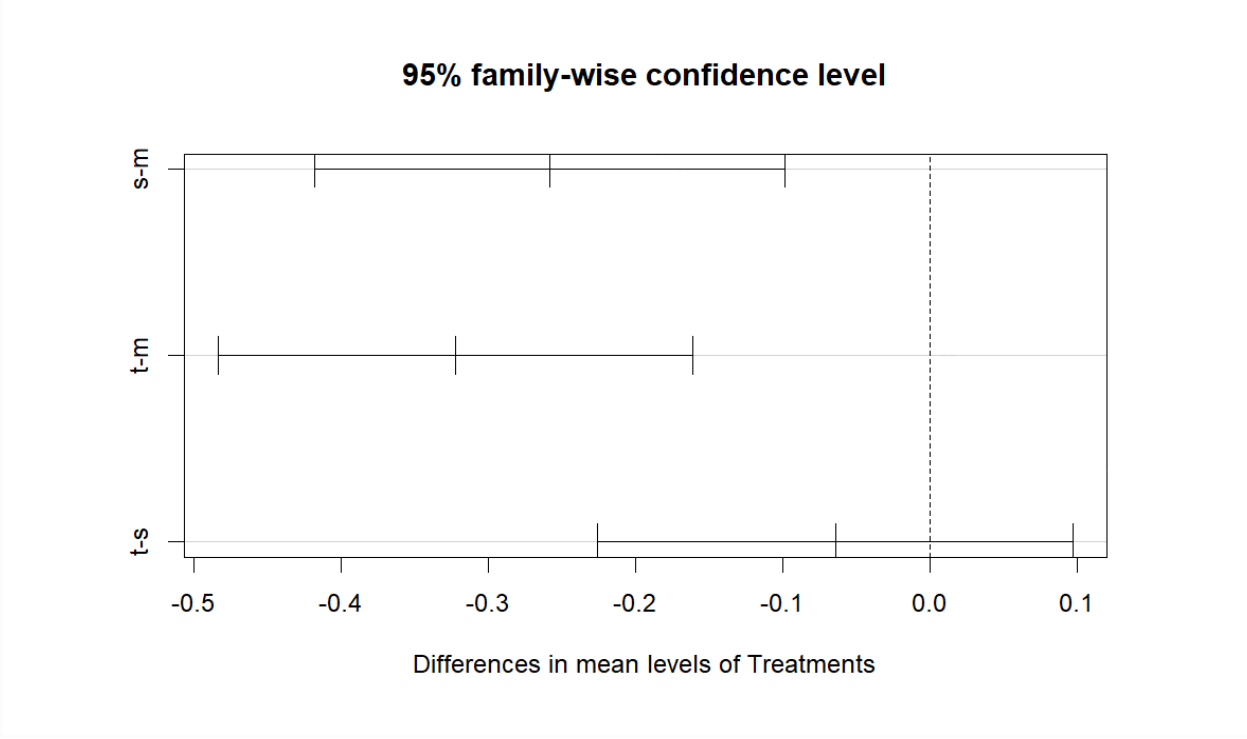


Figure 8. Tukey Test. Pairs between secondary and midrib, tertiary and midrib, and tertiary and secondary. There is a significant difference between t-m and s-m, but there is little difference for t-s.

DISCUSSION

Latex exudation measurements were taken for the midrib, secondary, and tertiary for all the different treatments and allowed for comparisons to be drawn between hierarchies, damage responses, and species. This consistency and wide range of species provided a greater generalizability to be drawn from these results than previous studies. The relationship between laticifers and vein hierarchy was drawn from a visual assessment of latex presence or absence., 100% of midrib and secondary veins had latex exudation, 96% of tertiary veins had latex exudation. Latex exudation averages were used to approximate vein scaling for midrib, secondary, and tertiary/lamina veins. Damage responses were calculated for the proximal midrib damage and whole trench damage by studying distal exudation amounts in relation to undamaged sites. Calculating the averages in percentages allowed for greater applicability among species and hierarchies.

Latex presence and vein scaling

Laticifer presence and latex exudation volume is directly related to vein hierarchy size. Laticifer cells are specialized to synthesize and accumulate latex and they form a tubing system in living plants (Castelblanque et al. 2016). In the midrib, the largest hierarchy tested, latex was present in all species tested. In the secondary vein hierarchy, latex was present in all species tested. In the tertiary/lamina hierarchy grouping latex was not always present. Latex was not found in the tertiary/lamina of 1/28 tested species. This pattern in the presence of latex can be further explained by the differences in exudation volume after cutting. For comparison the latex exudation amounts for the three hierarchies tested the average exudation volume of the 29 species (28 for tertiary/lamina) tested that was used as a generalizable number. It is found that on average 4.591 mm^3 latex was exuded from the midrib, 1.084 mm^3 of latex was exuded from the secondary vein hierarchy, and 0.294 mm^3 of latex was exuded from the tertiary/lamina hierarchy grouping. This indicates that latex exudation volume can be scaled with the vein hierarchy size. Roughly 4 times as much latex is exuded from the midrib to the secondary, and roughly 3.5 times as much latex is exuded from the secondary to the tertiary/lamina. In general, more latex is exuded from larger hierarchies (midrib > secondary > tertiary/lamina). Not only latex follows

this scaling trend, conduit diameters narrow from the petiole to the midrib to the secondary veins (Coomes et al. 2008). This narrowing reduces the dependency of hydraulic resistance on path length (Becker et al. 2000).

These comparisons were drawn using averages, and it was observed in species such as *Euphorbia socotrana* that these conclusions are not true. In *Euphorbia socotrana* it was measured that the tertiary/lamina hierarchy exuded more latex than the secondary vein hierarchy. These results, while generalizable to a wide variety of latex producing species, cannot be considered as a fact for all species that produce latex.

Latex damage response

The results of this thesis show that when one part of the leaf's laticifer network is damaged, the latex exudation at another location changes. The influence of proximal damage to latex exudation at distal hierarchies was tested in this study to simulate the proximal vein cutting behavior of certain insects to sever veins and block the flow of latex to feeding sites (Clarke and Zalucki 2000, Dussourd and Denno 1991, Dussourd and Eisner 1987, Helmus and Dussourd 2005). This can be analyzed by comparing proximal midrib damage and distal hierarchy damage as well as by comparing distal hierarchy damage to singular hierarchy damage. The average proximal midrib exudation was 6.981 mm^3. Latex volume decreased by 89.51%, 6.249 mm^3 at the distal midrib when compared to the proximal midrib latex exudation. Latex volume decreased by 98.71%, 6.891 mm^3 at the distal secondary vein hierarchy when compared to the proximal midrib latex exudation. Latex volume decreased by 99.42%, 6.941 mm^3 at the distal tertiary/lamina hierarchy when compared to the proximal midrib latex exudation. This demonstrates that damage done at one location in a leaf does impact the exudation at another location.

Comparison can be drawn between hierarchies of the same species when tested without damage to those that experience proximal midrib damage. On average latex exudation in the distal midrib was measured to be less than the undamaged midrib by 84.05%, 3.859 mm^3. On average latex exudation in the distal secondary vein hierarchy was measured to be less than the undamaged secondary vein hierarchy by 91.69%, 0.994 mm^3. On average latex exudation in the distal tertiary/lamina was measured to be less than the undamaged tertiary/lamina by 86.39%,

0.254 mm^3. This demonstrates that proximal midrib damage decreases the amount of latex exuded at a distal hierarchy.

These comparisons are drawn using average exudations of hierarchies across all species that were tested. Latex exudation at one location is influenced by damage at another location. It was also observed that many species experienced no exudation at distal hierarchies after the initial proximal midrib damage.

Latex whole trench damage response

Whole trench damage was tested in 24 species of latex producing plants. Whole trenching of the proximal vein hierarchies decreased the distal latex exudation. This whole trench proximal damage was done to simulate the trenching behavior of certain insects to sever veins and block the flow of latex to feeding sites (Clarke and Zalucki 2000, Dussourd and Denno 1991, Dussourd and Eisner 1987, Helmus and Dussourd 2005). On average whole trench midrib latex exudation is less than undamaged midrib latex exudation by 94.09%, 4.32 mm^3. On average whole trench secondary vein hierarchy latex exudation is less than undamaged secondary vein latex exudation by 88.83%, 0.963 mm^3. On average whole trench tertiary/lamina latex exudation is less than undamaged tertiary/lamina latex exudation by 96.93%, 0.285 mm^3. These latex exudation measurements shows that whole trenching of the proximal vein hierarchies of a leaf, which mimics the trenching behavior of insects, leads to a decrease in the amount of latex exuded distal to the whole trench when compared to undamaged hierarchies.

Insect herbivory

To study the intricacies of plant defense one can observe the coevolution of herbivorous insects with laticiferous plants. When a latex producing plant is damaged the latex oozes out and coagulates upon exposure to air, which makes latex similar to a toxic glue (Agrawal and Konno 2009, Agrawal et al. 2008). Vein-cutting is an attribute of specialist herbivores and is an adaptation to counter plant defense systems (Dussourd and Eisner 1987). The decreased distal latex exudation amounts after proximal damage indicates that there is an evolutionary advantage to the vein-cutting and trenching behaviors of insects (Figure 6).

Limitations

This experiment acts as an indicator of how latex interacts across species and hierarchies. While the results of this experiment can be applied to a wide number of latex producing plants, it has some key methodological limits and limited generalizability. Primarily, while this experiment tests a wide variety of latex producing plants, all these plants were from the UC Berkeley Botanical Garden and were not grown in their natural habitat. This lack of natural environment means that any environmental conditions that would influence latex production could not be observed in this study. Additionally, due to the high number of leaves required for a single replication of this experiment, each species was only tested once to preserve the garden's living collections. Some species were not able to have all tests performed due to a lack of leaves. Any conclusions being drawn from this data are based on only a single individual and a single replication, which limits the reliability of the data. Furthermore, all the damage done in this experiment was performed with a razor blade for simulated insect herbivory, but this is by no means an exact replica of the behavior of herbivorous insects. Plant responses to herbivory are complex and problems with researchers' ability to mimic 'natural' plant responses is constantly called into question. In recent years especially, simulated herbivory is thought to fail to induce plant responses that are essential for the complex interactions between plants and insect herbivores (Hjältén 2008). This lack of accuracy when it comes to correctly activating the plant's damage response could lead to the wrong conclusion. Thus, any conclusions drawn about herbivory can only be loosely tied to the experimental results of this study.

Future directions

Replicating the trials and testing new species would be ideal for creating a wider generalizability and increased reliability. This would lead to a better understanding of the trends set out by this experiment. There are thought to be over 20,000 latex producing species in over 40 families, so testing families that were not touched on in this study would improve the results and their implications.

Finding a way to accurately visualize the laticifers of the wide variety of latex producing plants that were tested would advance the study of latex producing plants and further the results of this experiment. The inability to visualize the laticifers in relation to leaf veins was a major drawback of this experiment. Understanding the physical location of laticifers in relation to leaf veins would shed light on the mechanisms that underlie latex exudation and transportation. Visualization would also prove a powerful tool to help further understand the trend related to vein size scaling and latex exudation volume.

Broader implications

This project contributes to a larger body of research pertaining to leaf venation and laticifer networks. Understanding this relationship provides insights into the evolutionary relationships between the herbivorous insects that feed on laticifers and the evolution of latex exudation. Most importantly this experiment provides a generalizable method of measuring latex at various vein hierarchies and allows for comparisons to be drawn between a wide variety of species. Such a far ranging analysis has not been done for latex producing plants.

From the results of this study, it has been found that every location in a leaf that has a vein also has a laticifer. Out of the 29 different species tested all the midrib and secondary vein locations have latex exudation. Of the 28 species tested for tertiary/lamina there was only 1 species with no latex exudation. Additionally, if the correlation of vein size to laticifer size is correct; larger veins have larger laticifers and smaller veins have smaller laticifers. Larger veins/laticifers lose higher amounts of latex than minor veins/laticifers. Furthermore, proximal vein cutting and damage on a leaf decreases the amount of latex exuded at the distal end across different hierarchies. This response is seen for both proximal midrib damage and proximal whole trench damage. Lastly, the trenching behaviors of insects provide an evolutionary advantage by decreasing distal latex exudation.

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APPENDIX

https://drive.google.com/drive/folders/1pfvaQ7ehc-zDQ0E_YKYuOOYwhzApDXaV